bioRxiv beta

THE PREPRINT SERVER FOR BIOLOGY

Within the fortress: A specialized parasite of ants is not evicted

Emilia S. Gracia, Charissa de Bekker, Jim Russell, et al.

1   **Within the fortress: A specialized parasite of ants is not evicted**

2   Emilia S. Gracia[1], Charissa de Bekker[2], Jim Russell[3], Kezia Manlove[4], Ephraim Hanks[3]

3   and David P. Hughes[2]

4   [1]Ecology, Department of Entomology and Center for Infectious Disease Dynamics, Pennsylvania State

5   University, University Park, State College, PA 16801

6   [2] Department of Entomology and Department of Biology, Center for Infectious Disease Dynamics,

7   Pennsylvania State University, University Park, State College, PA 16801

8   [3] Department of Statistics, Center for Infectious Disease Dynamics, Pennsylvania State University,

9   University Park, State College, PA 16801

10   [4] Department of Biology, Center for Infectious Disease Dynamics, Pennsylvania State University,

11   University Park, State College, PA 16801

12   **Abstract**

13   Every level of biological organization from cells to societies require that composing units come

14   together to form parts of a bigger unit (1). Our knowledge of how behavioral manipulating

15   parasites change social interactions between social hosts is limited. Here we use an endoparasite

16   to observe changes in social interactions between infected and healthy ants, using trophallaxis

17   (liquid food exchange) and spatial data as proxies for food sharing and social segregation. We

18   found no change in trophallaxis (p-value=0.5156). By using K-function and nearest neighbor

19   analyses we did see a significant difference in spatial segregation on day 3 (less than 8

20   millimeters; p-value<0.05). These results suggest healthy individuals are unable to detect the

21   parasite within the host.

22   **Key words-** Social parasite, individual behavior, *Ophiocordyceps unilateralis*,

23   *Camponotus castaneus*, behavioral manipulation, kin selection

24   **Introduction**

25   Every level of biological organization from cells to societies require that composing units come

26   together to form parts of a bigger unit (1). Where the composing units are themselves

27   individuals, as in the case of social insects, the success of the group requires these units to be

28   altruistic; explaining this behavior is conceptually challenging because one animal provides a

29   benefit to another at a cost to itself. The seminal contribution of W.D. Hamilton (2, 3) provided a

30   simple yet powerful framework for understanding altruism. This has since become known as

31   Hamilton's Rule and posits that a behavior or trait will be favored by selection, when $rb-c>0$,

32   where c is the fitness cost to the actor, b is the fitness benefit to the recipient, and r is their

33   genetic relatedness. Since then numerous studies across a very diverse range of organisms from

34   bacteria and yeasts to ants and mammals have demonstrated that the necessary conditions for

35   altruism are upheld (4, 1).

36   Crucial to understanding the evolution of altruism has been determining how animals

37   distinguish kin from non-kin because r must be >0 to satisfy Hamilton's rule. An unlikely tool

38   for studying altruism, it turns out, are parasites, the very antithesis of altruistic behavior.

39   Parasites have evolved ways to break the code within kin groups, benefiting from their altruism,

40   despite being completely unrelated to the donor (r=0). The classical example is the Common

41   Cuckoo (*Cuculus canorus*) that exploits the parental behavior of other bird species through egg

42   mimicry and selfish behaviors by the chick. In societies such as ants, where altruism is expressed

43   from sibling to sibling, diverse parasites ranging from other ants to beetles, flies, caterpillars and

44   even mollusks have evolved ways to break the code to act like cuckoos (5,6). For example,

45   caterpillars (i.e. *Maculinea rebeli*) perfectly mimic the chemical profile of larval ants and are

46   carried into the nest by foraging workers, where they are then fed colony resources and consume

47   the larval and egg stage ants (5,7,8,9). The general term for such organisms is social parasite and

48   studying their chemical ecology and behavior has provided many insights into the mechanisms

49   by which altruism works.

50   Although the color pattern of a cuckoo egg or the chemical cues of caterpillars entering ant nests

51   are complex, it is conceptually easy to imagine how they evolved from 'so simple a beginning'

52   (10). Indeed Darwin considered both cuckoo and socially parasitic ants, conjecturing that each

53   emerged from non-parasitic progenitors (Chapter 7). More difficult to conceptualize is whether a

54   parasite entering the body of an altruist can be recognized as a parasite since it is within the body

55   of a colony member who presents kin recognition cues to the rest of the colony. Here test

56   whether uninfected members of an ant colony can recognize siblings infected by a specialized

57   endoparasite. We will use as our model the entomopathogenic fungus, *Ophiocordyceps*

58   *unilateralis,* a highly specialized parasite of worker ants that manipulates host behavior to

59   achieve transmission.

60   In recent years a number of studies have demonstrated that species within the complex *O.*

61   *unilateralis s.l.* infect worker ants and adaptively manipulate host behavior by causing infected

62   individuals to leave the colony and bite into vegetation before dying (11). The function of such

63   manipulation is to provide a platform for spore release as post-mortem the fungus transitions

64   from growing with the body to growing externally, forming a large stalk from which spores are

65   produced and released onto the forest floor (12). Because the time from exposure and infection

66   to behavioral manipulation is between 9 days and 3 weeks, during which time the infected ant is

67   within the colony, this model offers the potential to examine how the colony responds to infected

68   individuals. In this study we develop a system for within colony observation of infected and

69   healthy individuals. We show that despite their status, infected ants are neither evicted from the

70   colony nor prevented from leaving the nest to die and spread disease to their siblings.

71   **Results**

72   **Infected ants receive food from siblings**

73   We first set out to determine if infected ants received food from their siblings inside the nest

74   area. We infected ten ants per colony in four different colonies of *Camponotus castaneus* species

75   with a strain of *Ophiocordyceps unilateralis* fungus, which naturally infects this species in the

76   wild (13). Ten ants were removed from the stock colony and injected with 1µl *O. unilateralis* in

77   solution with Grace's insect media supplement with 10% fetal bovine serum (FBS). A further 10

78   ants were sham treated, and inject with 1µl of Graces +FBS media. Both infected and sham

79   treated ants were maintained together with 15 additional untreated individuals in a wooden

80   chamber of volume 14.93 ± 0.53 cm^3 placed within a cage of 451.61cm^2 that served as a foraging

81   area and contained sand. These ants were given water and 10% glucose *ad libitum.* We began

82   continuous data recording from the third day post injection until day 18, moment at which there

83   was the least amount of infected individuals alive. Ant behavior was recorded inside the nest

84   with GoPro Heron 2 cameras for 24 hours/day. We then scored behavior from playback on

85   screens. We first focused on food exchange between individuals since out hypothesis was that

86   infected ants receive food at a different rate from non-infected ants. Worker ants cannot eat solid

87 food but instead exchange liquids in a process called trophallaxis. We followed 17 focal
88 individuals from one colony for a total observation 976.24 hours. Using a mixed effect linear
89 model, programmed in R, we used trophallaxis duration as a function of day post infection and
90 using ant identification as a random effect we found no significance *p-value*=0.5156. We found
91 no significant patterns in differences in either duration or count of trophallaxis (Figure 1). Our
92 quantification of observations includes only within nest exchange of liquids. We did however
93 also observe that ants infected by *O. unilateralis* would receive trophallaxis from nest mates
94 when outside the nest. Individuals were even fed in the minutes and hours before they were
95 behaviorally manipulated to ascend vegetation before biting bite into the twigs we provided and
96 dying. We therefore found no evidence that infected ants were refused food from other colony
97 members.

98 **Infected ants are not attacked by siblings**

99   It might be expected that infected ants are attacked more often. Although an infected ant can
100 only infect other ants following its own death and the subsequent growth of the stalk from its
101 head, it is possible that the increase of fungal cells within the worker ants changes some aspect of
102 it phenotype, such as smell, causing other ants to attack it. Because aggression might be rare and
103 fleeting we observed the behavior of 2 colonies (30±5 ants/colony) 24 hours/day for 18 days
104 (still in progress). We saw no aggression between untreated control ants and either infected or
105 sham treated ants. We conclude based on continuous observations over the entire course of
106 infection that infected ants are not attacked by their siblings.

107 **Infected ants are initially distanced from colony members but this declines over time**

108 Although we found no aggression there were subtle indications of infected ant segregation. To
109 measure this we applied spatial point process approaches to within-nest ant locations. To
110 examine spatial interaction behavior between healthy and infected ants, we examine the K-cross
111 function (14) between healthy and infected ants, which is the expected number of infected ants
112 that would be found within a distance *d* of each healthy ant.

113 Our null hypothesis is that ant infection status does not affect the tendency to group together or
114 avoid other ants. We used a nonparametric permutation test (15) to test for significant deviation
115 from this null behavior. In Figure 2 we show the observed K-cross function (in red) for all the

116 data, as well as for days 3, 6, and 9, together with 1000 K-cross functions (in black) simulated
117 from the null model by randomly permuting the labels (e.g., healthy, infected, or sham) of the
118 ants 1000 times, and calculating the K-cross function between healthy and infected ants under
119 each of these permutations. Significant deviation from the null model is indicated by an
120 observed K-function that lies on the edges (tails) of the envelope of K-functions simulated under
121 the null model, and empirical *p*-values can be computed by considering the rank of the observed
122 K-function within the envelope. Significant deviation from null behavior only occurs day 3 at
123 short spatial distances (less than 8 millimeters; p-value<0.05), where the observed K-function
124 lies on the lower tail of the permuted K-functions, suggesting spatial segregation at small spatial
125 scales.

126 To examine this potential small-scale ant interaction behavior between healthy and infected ants,
127 we found the nearest neighbor to each ant at each time point. We then tested for deviation from
128 the null assumption that ants are equally likely to have any other ant as nearest neighbor by again
129 permuting the ant labels at each time point and recording the permuted label of each ant's nearest
130 neighbor. Table 1 shows the proportion of healthy ants with an infected nearest neighbor over
131 all observed days, and for day 3, day 6, and day 9, together with the empirical *p*-value under the
132 null, obtained using 1000 permutations of ant labels at each time point of observation. We see
133 significant differences on day 3 and when we pool all the data together.

134 **Discussion**

135 Our data suggests no aggression towards infected individuals. We also see no distinct differences
136 in the mean duration or counts of trophallaxis between infected and uninfected individuals (p-
137 value=0.5156). Our linear mixed effect model shows trophallaxis had no relation with treatment
138 and trophallaxis is stochastic. Other studies have used trophallaxis as a tool to study social
139 immunity, making similar observations to the ones we have made here, yet their results are an
140 increase the amount of trophallaxis that occurs 24 hours after infection with a fungal pathogen
141 (16, 17). An important factor these papers did not take into account is time, they only made
142 observation 24 hours after the infection, this experiment on the other hand observed the ontogeny
143 of behavior within the nest. By using a one chamber scenario in a cage where ants were able to
144 freely move and interact with one another enables us to observe more naturalistic interactions
145 that has been lacking in the ant-pathogen research.

146   Our data suggest there are no shifts in behavior towards infected individuals, suggesting healthy

147   individuals are unable to detect *Ophiocordyceps* infection. The spatial point process analysis

148   revealed that by and large there is no evidence for spatial segregation of infected ants. The only

149   exception was the slight differences in spatial segregation between healthy individuals and those

150   infected at small spatial scales on day 3 of the infection, but not on days 6 and 9. These minute

151   changes in spatial arrangement could be caused by changes in individual infected ant behavior

152   and are not likely to be indicators of social exclusion, which we would expect to increase in

153   strength with time from infection. We did not test for any relationship between spatial

154   segregation and the identity of the focal individuals in relation to who perform the most

155   trophallaxis. Data collection for both trophallaxis and distance data were collected on Colony 2

156   the sample sizes we have may be masking the effect of *Ophiocordyceps* on the infected

157   individuals.

158   Within nest distance observations has been done before by using images to determine spatial

159   fidelity and time budgets of *Leptothorax acervorum* (18, 19), their observations did not take into

160   account how pathogens may change social dynamics within a colony nor did they do continuous

161   behavioral observations. We were able to observe rare interactions and behaviors that have

162   previously not been described. Being able to follow individuals through time and space lends

163   itself to be a powerful tool for further understanding the ontogeny of behavior within infected

164   individuals. Although our trophallaxis and distance results were not significant we can still

165   progress our understanding between uninfected individuals and those being parasitized.

166   In order to establish if these results are caused by the evolutionary history between

167   *Ophiocordyceps* and host we should observe non-coevolved pathogen species. These behavioral

168   assays enable us to further explore the role of parasites in not only the behavioral of the single

169   host, but also in the colony host. The ability to combine behavioral observation and spatial

170   dynamics as a tool to make very fine detailed observations enables us to further tease out the

171   dynamics of the colony and those infected. Another powerful tool that we could add to this type

172   of behavioral assay is chemical cues, such as cuticular hydrocarbons.

173   Chemical communication is the method of communication within an ant colony (20,21).

174   Therefore individual odor changes could signify caste allocation (22) and colony members could

175   also use it as a methods to determine health. Using continuous, detailed observations and

176 cuticular hydrocarbons would give insight into how these infected individuals are perseived by
177 their nest mates.

178

179 **Methods and Materials**

180 **Ant collection and stock colony maintenance**- Ants were collected in South Carolina during
181 October 2012. Colonies were collected by following foragers to nest sites that were then dug up.
182 Colony 1 consisted of sexuals, brood and about 120 workers; collected 10/4/2012. Colony 2
183 formed by 100 workers and brood; collected 10/5/2012. Colony 3 formed by100 workers and
184 brood; collected 10/3/2012. Colony 4 has queen, brood and 120 workers. Colonies were
185 maintained by providing them sugar water and water ad libitum and changed once a week. From
186 these colonies we collected individuals to run our experiment on.

187 **Infection techniques**- O. unilateralis Infections were done as described in de Bekker *et al*.,
188 2014/submitted. Single fungal colonies were placed in a sterile 2 mL tube with two 8/32 inch
189 metal balls (Wheels Manufacturing Inc.) and 200 µl Grace's medium (Sigma) freshly
190 supplemented with 10% FBS (PAA laboratories Inc.). The colony tissue was lysed using
191 TissueLyser II (Qiagen) at room temperature for 60 sec. at 30 freq/sec. This processed enabled
192 us to obtain single hyphae used at a mean concentration of $3.9x107+/- 1.1x10^7$ hyphae/ml for
193 infection. Infections were done by injecting 1 µL hyphal solution with a laser pulled 10 µL
194 micropipette (Drummond) and aspirator tube (Drummond) into the thorax underneath the front
195 legs. Sham treatments were done in similar fashion using 1 µL medium without hyphae (23).

196 **Treatments and individual identification-** Subcolonies were made of fifteen healthy, ten
197 injected with Graces+FBS media used for *Ophiocordyceps* growth in the laboratory and another
198 ten were injected with *Ophiocordyceps* plus media. These individuals were collected by their
199 colonies by agitating the housing tubes within each colony had and collect the individuals that
200 from the population. In order to follow individuals through time we used a dot system, each
201 individual had a different dot pattern painted on its body. We used an edding® 751 paint marker
202 to label the ants we used for the experiment.

203 **Behavioral observation set up**- We created sub-colonies containing 35 worker ants within a
204 wooden cage with a volume of $14.93± 0.53$ cm^3. In order to make 24 hour observations we used

205 a Go Pro camera (Hero 2 with IR lens) and an IR lamp was used for nocturnal observations. The

206 camera was located on top of the colony chamber and removed to change size video card three

207 times a day.

208 **Trophallaxis**- There was only one observer who made the observations of trophallaxis to reduce

209 observer bias. Trophallaxis was classified as starting when labrum was exposed and distended

210 between the two individuals. The event was as over when the mouth parts separated and the

211 individual parted ways. We observed a total of 976.24 hours of video for Colony 2 in order to

212 determine the amount of trophallaxis focal individuals were receiving on days 3-9,12,15 and 18

213 in trial one. A total of seven Infected individuals and five sham treated and five healthy ants

214 were followed over the course of the daylight session (7.68±0.32 hours per day) on days

215 3,4,5,6,7,8,9,12,15 and 18 post injection. We analyzed days 3-9 since these are the days we have

216 most infected individuals inside the nest (Figure 1). The chambers in which ants were placed did

217 not restrict individuals to stay within the nest, we were only able to record behaviors for those

218 present within the nest at the time of observation.

219 **Aggression**- There was only one observer who took note of aggressive behavior to reduce

220 observational bias. The videos were observed in fast forward and stopped if any abnormal

221 behavior occurred. Colony 2 has a total of 76.77 hours observed and no aggression has been

222 seen. Further observations will be made in other colonies to see if non-aggression holds.

223 **Distance data collection-** Screen shots were made for every ten minutes of observation during

224 the day period (8.24±0.34 hours per day). Individuals were identified using paint marks. We

225 then used an R program (version 2.15.1; created by Kezia Manlove) that calculated both pair-

226 wise distances and x-y coordinates for the individuals within the chamber. On average there were

227 24±2 individuals inside the chamber, we recorded point distances on all the individuals visible to

228 us on days 3, 6, 9 and 12 for a total of 4,758 x-y coordinates and 61,000 pair-wise data points.

229 **Distance data analysis**- We focused on days 3, 6 and 9 (8.09±0.43 hours per day) when

230 analyzing the data. We used point process models, which take a set of point locations in window

231 of space. Each of these points can be labeled with a mark to indicate a certain type or class. In

232 this case, the ant locations are the points of interest, the window of observation is the nest, and

233 the mark of each ant is their infection status; either untreated, infected, or sham treated. In point

234 process statistics, the interaction between points can be measured using a summary statistic

235 called the K-function (14, 15). For a given distance *d*, the K-function gives the expected number

236 of additional ants to be found within a radius of *d* of a focal ant.

237 Functions to compute the K-cross function from healthy to infected ants, and the nearest

238 neighbor to each ant at each time point, were created in R. The K-cross function finds the

239 average number of infected ants within a specified distance of a healthy ant, with the average

240 being over all healthy ants in the chamber at each time point, and over all time points within the

241 specified day. The permutation tests for the nearest-neighbor analysis were carried out by

242 permuting the labels (healthy, infected, or control) of ants in the chamber at each time point and

243 re-computing the nearest neighbor of each ant.

250 **Refrences**

251 1. Bourke AFG (2011) Principles of Social Evolution. *Oxford, Oxford University Press*.

252 2. Hamilton WD (1963) Evolution of Altruistic Behavior. *American Naturalist*, **97**(896):354-
253 356

254 3. Hamilton WD (1964) The genetical evolution of social behaviour. I & II. *Journal of*
255 *Theoretical Biology*, **7**:1-52.

256 4. Foster KR, Wenseleers T, Ratnieks FLW (2006) Kin selection is the key to altruism. *Trends*
257 *in Ecology & Evolution* 2006, **21**(2):57-60.*

258 5. Guillem RM, Drijfhout F, Martin SJ (2014) Chemical deception among ant social parasites.
259 *Current Zoology*, 60**.**

260 6. Foitzik S, DeHeer CJ, Hunjan DN, Herbers JM (2001) Coevolution in host–parasite systems:
261 behavioural strategies of slave–making ants and their hosts. *Proceedings of the Royal Society*
262 *of London Series B: Biological Sciences*, **268**(1472):1139-1146.

263   7. Als TD, Vila R, Kandul NP, Nash DR, Yen S-H, Hsu Y-F, Mignault AA, Boomsma JJ,
264   Pierce NE (2004) The evolution of alternative parasitic life histories in large blue butterflies.
265   *Nature*, **432**(7015):386-390.

266   8. Nash DR, Als TD, Maile R, Jones GR, Boomsma JJ (2008) A mosaic of chemical
267   coevolution in a large blue butterfly. *Science*, **319**(5859):88-90.

268   9. Gilbert OM, Foster KR, Mehdiabadi NJ, Strassmann JE, Queller DC (2007) High relatedness
269   maintains multicellular cooperation in a social amoeba by controlling cheater mutants. *PNAS*,
270   **104**: 8913-8917.

271   10. Darwin C (1859) On the Origin of Species by Means of Natural Selection, or the
272   Preservation of Favoured Races in the Struggle for Life. *London: John Murray*.

273   11. Hughes DP, Andersen S, Hywel-Jones NL, Himaman W, Billen J, Boomsma JJ (2011)
274   Behavioral mechanisms and morphological symptoms of zombie ants dying from fungal
275   infection. *BMC Ecology*, **11**: 13.

276   12. Andersen, SB, S. Gerritsma, K.M. Yusah, D. Mayntz, N.L. Hywel-Jones, J. Billen, J.J.
277   Boomsma, D.P. Hughes (2009). The life of a dead ant – the expression of an extended
278   phenotype. American *Naturalist* 174: 424–433

279   13. Evans HC, Elliot SL, Hughes DP (2011). *Ophiocordyceps unilateralis. Communicative &*
280   *Interactive Biology*, **4**: 5, 598-602.

281   14. Gaetan, C. and X. Guyon (2010). Spatial Statistics and Modeling. *Springer*.

282   15. Givens, G.H. and J.A. Hoeting (2012). Computational Statistics. *John Wiley and Sons*.

283   16. Hamilton C, Lejeune BT, Rosengaus BR (2011). Trophallaxis and prophylaxis:social
284   immunity in the carpenter ant *Camponotus pennsylvanicus*. *Biology letters*, **7:** 89-92.

285   17. de Souza DJ, Vlaenderen JV, Moret Y, Lenoir A (2008). Immune response affects ant
286   trophallactic behavior. *Insect Pathology*, **54**, 828-832.

287   18. Sedova-Franks AB, Franks NR (1995). Demonstrating new social interactions in ant
288   colonies through randomization tests: separating seeing from believeing. *Animal behavior*,
289   **50**, 1683-1696.

290   19. Franks NR, Bryant S, Griffiths R. Synchronization of behavior within nests os the ant
291   *Leptothorax acervorum* (Fabricius)- I. Discovering the phenomenon and its relation to the
292   level of starvation. *Bulletin of mathematical biology*, **52**:5, 597-612.

293   20. Hölldobler,B. and Wilson, E.O (1990) The ants.Cambridge, MA:Belknap Press, Harvard
294   University.

295   21. Hölldobler,B. and Wilson, E.O (2009) The Superorganism. London: WW Norton.

296   22. Greene MJ, Gordon DM (2007). Interaction rate informs harvester ant task decisions.

297   *Behavioral Ecology*, **18**(2), 451-455.

298   23. de Bekker C, Quevillion L, Smith PB, Fleming K, Ghosh Debashis, Patterson AD, Hughes

299   DP (2014) Species specifi ant brain manipulation by a specialized fungal parasite In review

300

301   **GRAPHS AND TABLES**

302

303   **Figure 1**- Trophallaxis data collected from videos on days 3-9, error bard represent the standard
304   error of the data. We were unable to see any significance difference between treated individuals
305   and healthy. (A) Shows no differences between infected, graces and healthy ants although we do
306   see an interesting pattern of duration increase on day 4 and 8. (B) Mean count of trophallaxis
307   changes slightly throughout the days we have observed. (C) The proportion of time spent in
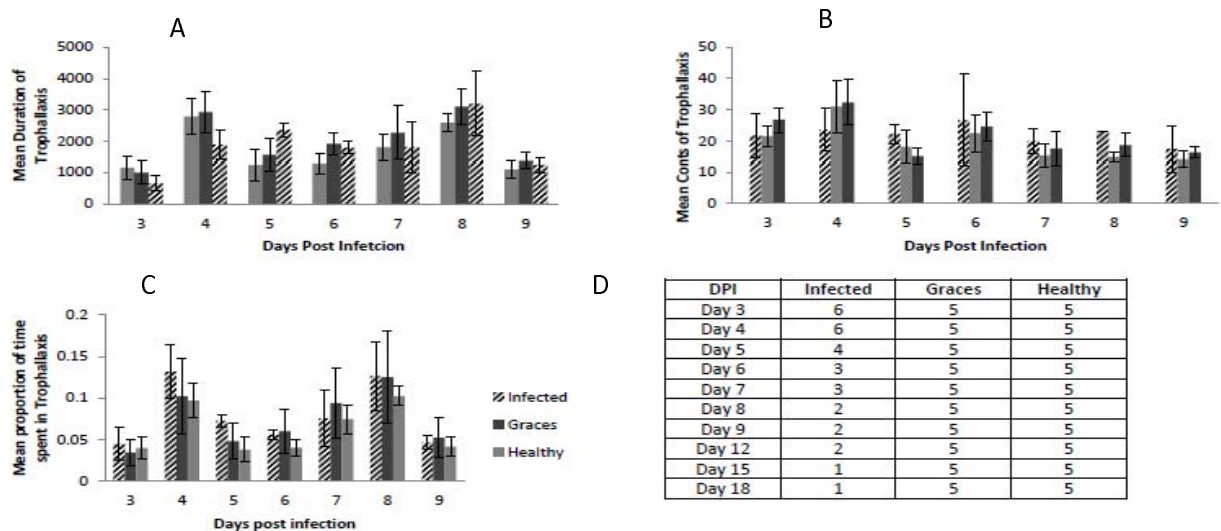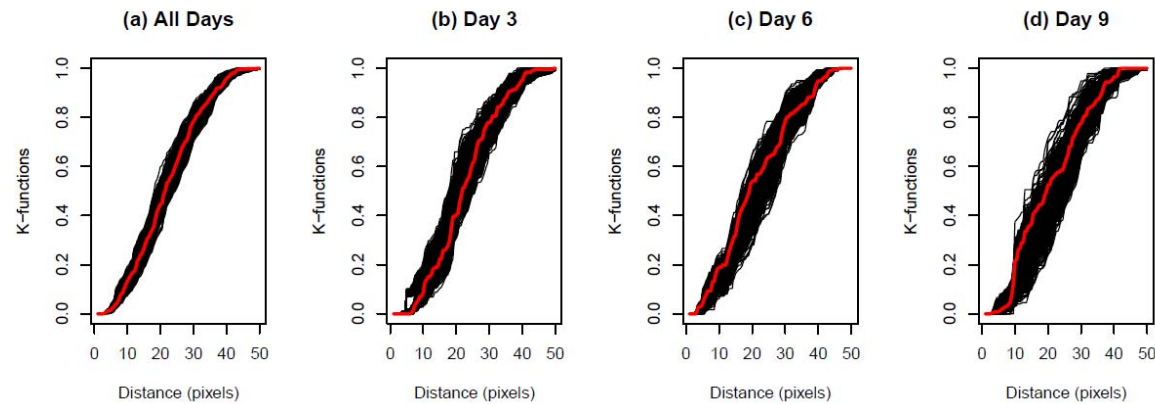308   trophallaxis.

DPI	Infected	Graces	Healthy
Day 3	6	5	5
Day 4	6	5	5
Day 5	4	5	5
Day 6	3	5	5
Day 7	3	5	5
Day 8	2	5	5
Day 9	2	5	5
Day 12	2	5	5
Day 15	1	5	5
Day 18	1	5	5

309

310   **Figure 2-** K function analysis

311

312

313

314   **Table 1-** Nearest neighbor analysis we can see there is a significant difference between healthy
315   and infected on when looking at all three days combined and only on day 3.

Time	Proportion	Permutation Test p-value
All Days	0.110	0.002
Day 3	0.108	0.004
Day 6	0.208	0.48
Day 9	0.104	0.24

316